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Denitrification by *Azospirillum brasilense* Sp 7

I. Growth with nitrite as respiratory electron acceptor

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Abstract. *Azospirillum brasilense* Sp 7 grows anaerobically both in batch and continuous culture utilizing nitrite as respiratory electron acceptor. Both nitrite concentration and pH are critical during growth. Nitrite is quantitatively converted to N_2 without the formation of any trace of free nitric oxide, nitrous oxide or ammonia. Nitrite is, however, converted to nitrous oxide in the presence of acetylene. Whereas nitrate reductase is always present under anaerobic conditions, the expression of nitrite and nitrous oxide reductase activities depends on the culture conditions. Nitrite respiration is poorly affected by carbon monoxide but severely by hydroxylamine which is not reduced by *Azospirillum*. It is suggested from the inhibitor studies that *Azospirillum* forms a cytochrome c, d type of dissimilatory nitrite reductase. In the absence of respiratory electron acceptors, *Azospirillum* utilizes malate to form poly-β-hydroxybutyrate (PHB). Denitrification is considered to be an undesirable feature in natural plant-*Azospirillum* associations under most conditions.

Key words: Denitrification – Dissimilatory nitrite reductase – Nitrate respiration – Nitrite respiration – *Azospirillum*

Azospirillum strains are abundant particularly in tropical soils and perform N_2-fixation in association with grasses. It had been suggested that *Azospirillum* supplies N_2-fixation products to cereals and that it may be of potential use for practical applications (Döbereiner and De-Polli 1980; Kapulnik et al. 1983). N_2-Fixation by *Azospirillum* was estimated to substitute for nitrogen fertilizers in the order of 10–20% under favorable conditions (Magalhães et al. 1978b). The picture may, however, be disturbed by the fact that *Azospirillum* also brings about denitrification. In the absence of O_2, the bacterium utilizes nitrate as terminal respiratory electron acceptor and converts it to gaseous nitrogen (Neyra et al. 1977; Eskew et al. 1977; Tarrand et al. 1978). It may, therefore, significantly contribute to losses of nitrogen in soils. Model experiments with wheat-*Azospirillum* associations, indeed, indicate that *Azospirillum* performs either N_2-fixation (C_2H_2-reduction) or denitrification, depending on the concentration of O_2 and nitrate in the assays (Bothe et al. 1983a; Bothe et al. in preparation).

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In denitrification, nitrate is reduced to nitrite and further to N_2O, N_2 or ammonia (Ingraham 1981; Whatley 1981). In the case of *Azospirillum*, recent experiments showed that this bacterium can even grow in liquid culture anaerobically with nitrate as respiratory electron acceptor (Bothe et al. 1981, 1983b). Under these conditions, the cells excrete large amounts of nitrite which can partly be reduced further to N_2O or N_2. This dissimilatory reduction of nitrite has only been described in qualitative terms up till now for *Azospirillum* (Magalhães et al. 1978a). The physiological function of nitrite reduction is unknown. It had been described for other bacteria that the dissimilation of nitrite can serve three different purposes: First, because high concentrations of nitrite are toxic, this compound is reduced to non-deleterious gaseous nitrogen, e.g. in propionibacteria (Kaspar 1982) or Citrobacter (Smith 1982). Second, fermentative bacteria, e.g. *Escherichia coli, Clostridium perfringens* or *Achromobacter fischeri* remove excess of reducing equivalents by forming ammonia from nitrite (Yordy and Ruoff 1981; Schlegel 1981). Third, the free energy change of the reduction of nitrite to N_2O, N_2 or ammonia is high enough to permit the generation of energy sufficient for growth (Thauer et al. 1977). Several nitrite respiring bacteria, e.g. *Pseudomonas denitrificans* (Bryan 1981) or *Vibrio succinogenes* (Bokranz et al. 1983) were, indeed, shown to grow under these conditions.

The present communication will describe that the third alternative is true for *Azospirillum brasilense* Sp 7. This strain grows anaerobically in batch and continuous culture provided the concentration of nitrite is kept low in the medium. Nitrite is quantitatively converted to molecular nitrogen under these conditions.

Materials and methods

Organism and growth

The type strain, *Azospirillum brasilense* Sp 7 (ATCC 29145) was used in all the experiments. Single colonies taken from nutrient agar plates (Difco) were grown in 100 ml Erlenmeyer flasks under sterile conditions containing the medium described by Bothe et al. (1981). For this medium, $FeSO_4$ (6.9 g/l) was dissolved with EDTA (9.3 g/l), sterilized separately and added after autoclaving (1 ml/l medium, final iron concentration 2×10^{-5} M). L-Malate and phosphate buffer were adjusted to pH 6.9 by KOH and autoclaved separately.

Growth in continuous culture (Table 1, Fig. 3) was performed by the chemostat method with malate limiting. In a typical experiment, a 16 l fermenter (Fermenter Kiel, Eschweiler & Co., Kiel, FRG) contained 5.2 l of the medium (Bothe et al. 1981) containing 2 mM $NaNO_2$ instead of KNO_3 and a reservoir where the medium contained 4 mM L(−)malate and 14 mM $NaNO_2$ at pH 5.0. The medium both in the reservoir and in the fermenter was continuously flushed with N_2 (rate ~38 cm^3 N_2/min). The fermenter was inoculated with 100 ml of the nitrate grown cells ($O.D._{560\,nm}$ ~1.7 corresponding to approximately 3×10^8 cells/ml). Fresh medium was continuously pumped into the culture with a peristaltic pump (dilution rate 0.03/h). Steady state was reached in about 72 h and could be maintained for at least 4 weeks. During steady state, the pH kept constant at 7.8, because the cells continuously utilized malate and because fresh medium with a pH-value of 5.0 was continuously added. The culture stayed at $O.D._{560\,nm}$ = 0.22 and malate was completely used up by the cells. Under these conditions, dissimilatory nitrite reductase activity was reproducibly high.

Table 1. Specific activities of nitrate, nitrite and nitrous oxide reductases in intact cells of *Azospirillum brasilense* Sp 7 grown anaerobically under different culture conditions

Culture condition	NO_3^--reductase	NO_2^--reductase	N_2O-reductase
1. Batch culture, pH 7.2 10 mM nitrate	12.3	0.2	0.2
2. Chemostat culture, pH 6.8, 10 mM nitrate	17.8	0.2	1.5
3. Chemostat culture, pH 8.0, 10 mM nitrate	7.5	4.6	3.6
4. Chemostat culture, pH 7.5, 10 mM nitrite	8.4	20.2	8.8
5. Chemostat culture, pH 7.6, 5.5 mM nitrite	5.5	8.1	15.0
6. Chemostat culture, pH 7.0, 5.5 mM nitrite	16.7	4.2	28.9
7. Batch culture, pH 7.2, 9 mM N_2O	12.6	2.4	28.6
8. Chemostat culture, pH 7.4, 5 mM NH_4^+, aerobic culture	7.3	0.2	1.5

Data are given in μmol NO_3^-, NO_2^- or N_2O utilized/h × mg protein. The concentrations of nitrogen compounds refer to the amounts in the reservoir in the case of the chemostat cultures and in the medium in the case of the batch cultures. The final concentrations of nitrite were in the case of culture 4: 0.48, culture 5: 0.03 and culture 6: 0.05 mM. The pH-values are always final ones in the culture. For other experimental conditions see Materials and methods

For nitrite dependent growth under anaerobic conditions in batch culture (see Fig. 1), 800 ml of the cells grown in continuous culture anaerobically with nitrite ($O.D._{560\,nm}$ = 0.65 corresponding to approximately 1.0×10^8 cells in this case) were inoculated into 8 l medium containing 2 mM $NaNO_2$ instead of KNO_3, 0.01 M L(−)-malate and all the other salts of the medium described previously (Bothe et al. 1981). The pH in the medium was always kept at 7.8 by adding HCl, and additional $NaNO_2$ was added upon exhaustion. Care had to be taken that the amount of nitrite never exceeded 2 mM. The medium was continuously flushed with N_2 from 1 h before the inoculation on and during growth. The concentration of O_2 was less than 0.05% in the gas phase throughout the experiment.

To ensure even more strict anaerobic conditions, *Azospirillum* was grown under sterile conditions in Thunberg tubes where the side arm contained 0.5 ml of a solution of 10% pyrogallol and 10% $NaHCO_3$ dissolved in water as an O_2 trap (see Fig. 2). The tubes were filled with 10 ml medium containing 37 mM D,L-malate, mineral salts (see Bothe et al. 1981), 8.5 mM K^+-phosphate buffer pH 7.2 and the different amounts of nitrite as indicated in the abscissa of Fig. 2. For the inoculum, *Azospirillum* grown in continuous culture anaerobically with nitrite was concentrated by centrifuging and suspending in the medium just mentioned. 0.2 ml of this suspension (corresponding to approximately

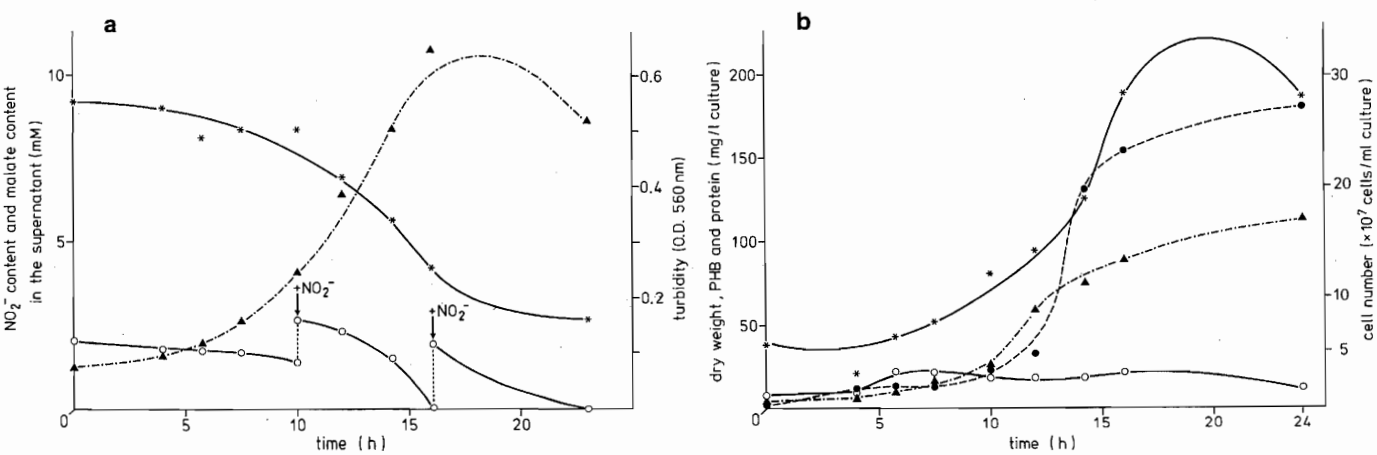

Fig. 1a, b. Nitrite dependent anaerobic growth of *Azospirillum brasilense* Sp 7 in batch culture. The experiment was performed in 8 l medium as described under Materials and methods. **a** ○——○ nitrite content in the supernatant (mM); *——* L-malate content in the supernatant (mM); ▲–·–·–▲ turbidity ($O.D._{560\,nm}$). **b** ●—— ● cell number (× 10^7 cells/ml culture); *——* dry weight (mg/l culture); ▲–·–·–▲ protein (mg/l culture); ○——○ poly-β-hydroxybutyrate (mg/l culture)

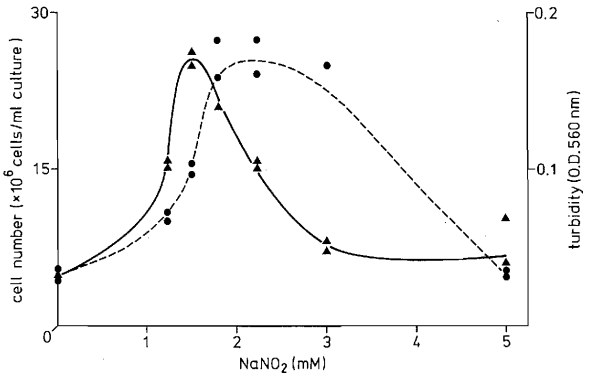

Fig. 2. Dependence of the anaerobic growth with nitrite as respiratory electron acceptor on the concentration of nitrite in the medium. The experiment was performed in Thunberg tubes as described under Materials and methods. ▲——▲ cell number ($\times 10^7$ cells/ml culture); ● --- ● turbidity

3×10^8 cells/ml) was pipetted into Thunberg tubes, and the incubation was performed for 48 h and 30° C under argon.

Analytical procedures

For determining nitrate, nitrite and nitrous oxide disappearance activities (Table 1, 3; Fig. 4), cells taken from the different cultures were washed by centrifuging at 10,000 × *g* for 10 min and 20° C and by suspending in the nitrogen free malate medium. All handlings were performed under anaerobic conditions as far as possible. 2.2 ml of these cells (concentrated to $O.D._{560\,nm}$ = 1.0 equalling approximately 0.2 mg protein/ml) were assayed in 7.0 ml Fernbach flasks under argon in a shaking water bath for 1–2 h at 30° C. The reactions were started by adding either 2.5 mM $NaNO_3$, 2.5 mM $NaNO_2$ or 9 mM N_2O (the latter concentration for the gas phase).

Nitrate was determined by nitration of salicyclic acid (Cataldo et al. 1975) and nitrite by the naphthylethylenediamine/sulphanilamide reagent (Neyra and van Berkum 1977). Poly-β-hydroxybutyrate was quantitatively measured as crotonic acid (Law and Slepecky 1961). Protein was determined by a modification of the Lowry method for intact cells (Herbert et al. 1971). For dry weight measurements, 400 ml cells were freed from the medium by centrifugation and washing with destilled water and dried at 80° C in alumina foil pots and placed overnight in a desiccator over silica gel. Cell numbers were counted in a Helber counting chamber. L(–)Malate was determined by converting it to citrate via oxaloacetate in the presence of coenzyme A, acetylphosphate and the enzymes malate dehydrogenase, citrate synthase and phosphotransacetylase and by measuring the NADH formed (Schoner 1970). Gas chromatographs fitted with thermal conductivity detectors served for the determinations of N_2O (Porapak Q column, He as the carrier gas) and of N_2 (molecular sieve 5 Å column, argon as the carrier gas).

Chemicals

Gases came from Linde AG, Höllriegelskreuth near München, salts from Merck, Darmstadt and enzymes from Boehringer, Mannheim.

Results

Azospirillum brasilense Sp 7 grows anaerobically in batch culture with nitrite as the respiratory electron acceptor (Fig. 1a, b). Growth under these culture conditions (see Materials and methods) commenced after a lag phase of approximately 5 h and proceeded exponentially for at least 10 h, when the concentration of nitrite was kept under 2 mM. Growth was strictly dependent on nitrite, indicating that the level of residual O_2 in the medium was too low to support respiration of the bacteria. Growth was monitored by turbidity, dry weight, cell number and protein content, and the curves were essentially in parallel during these 10 h. The pH in the medium was very critical during growth with nitrite, because the cells grew only between pH 7.4 and 8.2. Therefore the pH was measured every h and readjusted to pH 7.8 if necessary. During logarithmic growth, the specific growth rate was $\mu = 0.14$ h and the generation time was 3.8 h (taken from the curve for cell numbers). The two data are similar to those obtained in cultures grown with nitrate (Bothe et al. 1981, 1983b), O_2 (Volpon et al. 1981) or under N_2-fixing conditions (Volpon et al. 1981). Malate content continuously decreased in the medium, and exhaustion of this carbon source finally terminated growth (Fig. 1a). In this assay with optimal concentrations of nitrite in the medium, the cells did not store poly-β-hydroxybutyrate (PHB, Fig. 1b).

The concentration of nitrite in the medium was very critical (Fig. 2). In this experiment, the cells were grown in Thunberg tubes with different amounts of nitrite in the medium and with pyrogallol in the side arm to remove traces of oxygen. The data clearly show that growth was not due to residual O_2 in the tubes. The curves for cell number and turbidity were in parallel up to 2 mM $NaNO_2$ in the medium (Fig. 2). Above 2 mM $NaNO_2$ cell number decreased whereas turbidity stayed constant. This result shows that growth cannot always be monitored simply by measuring turbidity. Above 3 mM $NaNO_2$ both cell number and turbidity decreased (Fig. 2). Such high concentrations cause death of the cells.

Figure 3 gives a series of experiments with continuous cultures under chemostat conditions with malate limiting and where the concentration of nitrite was varied from continuous culture to continuous culture. When the amount of nitrite was low in the dilution medium (in the reservoir), the cell number in the culture was low, and the cells formed large amounts of PHB and very little protein. Cell number and protein content steadily increased upon raising the amount of nitrite in the dilution medium (about 3 mM nitrite in the culture). PHB-content of the cells decreased in a reciprocal way. When the concentration of nitrite in the dilution medium was above 8 mM, the cells did not utilize all the nitrite offered (Fig. 3). Under these conditions, they were apparently saturated with respiratory electron acceptors and did not store PHB any more. The data show that *Azospirillum* forms PHB from malate when respiratory electron acceptors are limiting. In all the different cultures, dry weight essentially remained constant. Thus the experiments show that nitrite respiration supports growth also in continuous culture.

Cells taken from the continuous culture were assayed for product formation of nitrite respiration (Fig. 4). Between pH 6.9 and 8.2 in the assays, *Azospirillum* quantitatively converted nitrite to N_2. Activities sharply decreased under

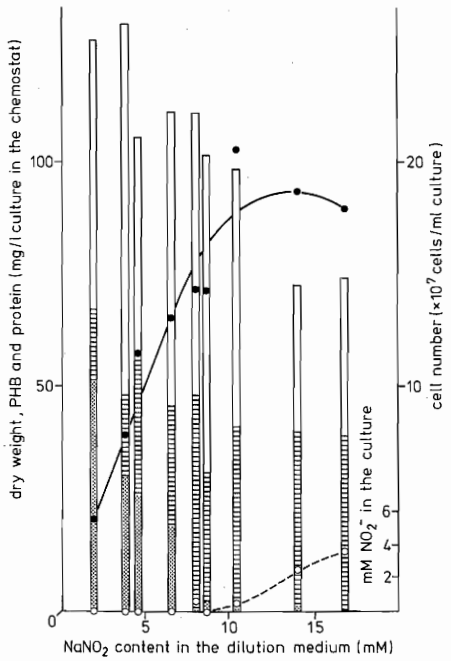

Fig. 3. Nitrite dependent anaerobic growth of *Azospirillum brasilense* Sp 7 in continuous culture under chemostat conditions. The conditions for establishing the continuous culture are described under materials and methods. The L-malate content in the reservoir was always 4 mM and the nitrite content varied from culture to culture. The concentrations given in the abscissa of the figure refer to the amounts of nitrite in the dilution medium (=reservoir). ●——● cell number ($\times 10^7$ cells/ml culture); ▨ poly-β-hydroxybutyrate (mg/l culture); ▥ protein (mg/l culture); ○ --- ○ NO_2^- in the supernatant of the culture; ▭ dry weight

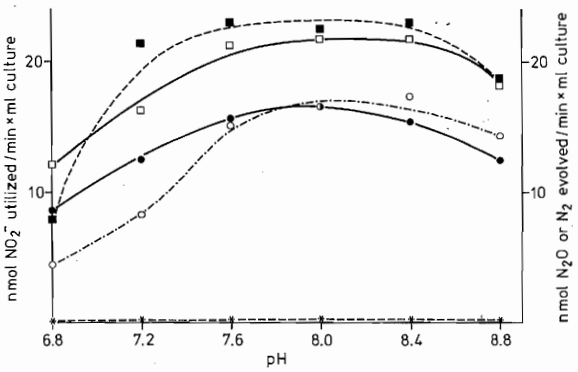

Fig. 4. pH-dependence of nitrite utilization and N_2 or N_2O formation by *Azospirillum brasilense* Sp 7. The cells taken from the fermenter grown anaerobically with nitrite were assayed in 7 ml Fernbach flasks containing 100 mM TES or Tricine-buffer pH as indicated in the figure, 2.2 ml cells ($O.D._{560\,nm}$ = 0.43) and 7 mM C_2H_2 in the gas phase where indicated. For other experimental conditions see Materials and methods. □——□ NO_2^--utilization; ●——● N_2-formation; ∗---∗ N_2O-formation; ■ --- ■ NO_2^--utilization (in the presence of C_2H_2); ○ -·-·- ○ N_2O-formation (in the presence of C_2H_2 in the vessel)

and above these pH-values. The stoichiometry between N_2-formation and nitrite disappearance was close to 1:2. The cells formed neither free nitric oxide (not documented) nor nitrous oxide (Fig. 4) nor ammonia (not documented). Nitrite was, however, converted to nitrous oxide when the gas phase contained C_2H_2 (Fig. 4). C_2H_2 was described to specifically block nitrous oxide reductase in denitrifying bacteria (Balderstone et al. 1976; Yoshinari and Knowles 1976), and this is also the case for *Azospirillum* (see accompanying paper).

Table 1 contains data for the enzyme activities in eight representative cultures chosen out of a large series of investigations. The specific activities were, admittedly, variable from culture to culture even under identical growth conditions (maximally by a factor of 2). This was mainly due to the fact that some activity may have been lost by taking the cells out of the fermenter and by concentrating and assaying them. N_2O-Reductase was particularly unstable which had also been observed with the enzyme from other organisms (Ingraham 1981). Nevertheless, the data allow to draw the following conclusions: Dissimilatory nitrate reductase was always demonstrable in the cells, regardless of whether grown anaerobically with nitrate, nitrite or nitrous oxide. Thus the expression of nitrate reductase activity is not caused by nitrate but the enzyme is always present under anaerobic conditions. In contrast, nitrite reductase and N_2O-reductase were not detected in *Azospirillum* in batch cultures grown anaerobically with nitrate and at pH-values around 7.0 in the medium, regardless of whether cells were assayed from the early or late logarithmic growth phase of batch cultures or from continuous cultures. Cells grown at pH 8.0 had, however, both enzyme activities at early stages of the logarithmic growth, though with rather low rates. N_2O-Reductase and nitrite reductase activities were expressed independently of each other and were strongly dependent on the pH-value in the medium. Cells were obtained which had high N_2O-reductase and low nitrite reductase activities and vice versa. The activities of nitrous oxide reductase were particularly high when the level of nitrite in the medium was low.

The effects of inhibitors were tested on nitrite reductase in cells grown anaerobically with nitrite as the respiratory electron acceptor (Table 2). The enzyme in the cells taken from the continuous culture were assayed by the disappearance of nitrite and by the formation of N_2O (in the presence of C_2H_2 in the vessels). Remarkably, both nitrite reduction and N_2O-formation were blocked up to 70% by 1 mM NH_2OH. The cells were also unable to reduce NH_2OH (not documented). NaCN ($K_i \sim 0.05$ mM) was inhibitory, whereas sodiumazid ($K_i \sim 5.0$ mM) and CO were poorly effective. 0.87 mM CO in solution corresponding to 100% CO in the gas phase caused an inhibition to only about 60% both with nitrite reduction and nitrous oxide formation (Table 2).

Discussion

Azospirillum had been described to utilize nitrite and to reduce nitrous oxide, but the studies were only performed qualitatively (Magalhães et al. 1978a). It was not clear whether nitrite had been reduced to N_2O, N_2, ammonia or a mixture of these nitrogen compounds. The present study shows that *Azospirillum brasilense* Sp 7 quantitatively converts nitrite to N_2, without the formation of either free nitrous oxide or ammonia. Product formation in denitrification of *Azospirillum* is thus similar as in most denitrifying bacteria, e. g. *Pseudomonas denitrificans* (Koike and Hattori 1975). Similarly, Rhizobium also performs nitrogen fixation

Table 2. Effects of several inhibitors on the dissimilatory nitrite reduction by *Azospirillum brasilense* Sp 7

Inhibitor	Concentration (mM)	NO_2^- utilization (% activity)	N_2O evolution (% activity)
1. Without inhibitor	–	100	100
2. NH_2OH	0.1	93	98
	0.5	65	64
	1.0	28	25
3. NaCN	0.001	98	96
	0.01	81	82
	0.1	12	10
4. CO	0.016	97	91
	0.16	78	64
	0.87	43	25
5. NaN_3	0.5	99	98
	5.0	30	10
	50	14	6

100% activity corresponds to 8.8 ± 1.4 µmol NO_2^- utilized or 4.2 ± 0.8 µmol N_2O formed/h × mg protein. The % data represent average values from at least three different experiments. The assays in the 7.0 ml Fernbach flasks contained 2% (= 0.83 mM) C_2H_2 in the gas phase in order to stop nitrite reduction at N_2O-formation. For experimental conditions see Materials and methods

and denitrifies nitrate and nitrite to N_2O (Zablotowicz and Focht 1979; Daniel et al. 1980) and to N_2 (Neal et al. 1983). In the presence of C_2H_2, denitrification of *Azospirillum* stops at the production of nitrous oxide as it does with most denitrifying bacteria. Unlike this, *Vibrio succinogenes* produces ammonia and does not form even a trace of nitrous oxide in the presence of C_2H_2 (Yoshinari 1980). Such examples show that the pathways of dissimilatory nitrate reduction can differ from organism to organism.

The present investigation also shows that *Azospirillum* can even grow anaerobically in liquid batch and continuous cultures utilizing nitrite as respiratory electron acceptor. Dissimilatory nitrite reduction is thus not merely a mechanism to detoxify or to remove excess of reductant but it can supply *Azospirillum* with enough energy for growth. In these growth experiments, the concentration of nitrite had always to be kept low. As other organisms, *Azospirillum* could not cope with high amounts of nitrite which then probably acted as reductant unspecifically.

The experiments also show that *Azospirillum* cannot reduce NH_2OH which, however, blocks nitrite reduction. Two different dissimilatory nitrite reductases have been described for denitrifying bacteria, a copper containing enzyme which reduces NH_2OH and a cytochrome c, d type protein which does not do so (Bryan 1981). The experiments with NH_2OH suggest that *Azospirillum* possesses the cytochrome c, d containing dissimilatory nitrite reductase. At the first glance, the observation may appear surprising that CO only poorly affects nitrite reduction. However, CO was described to block O_2-linked terminal respiratory oxidase but not dissimilatory nitrate reduction also in other denitrifying bacteria, e.g. *Paracoccus denitrificans* (Payne 1973).

In the absence of respiratory electron acceptors including nitrite, *Azospirillum* utilizes malate and forms poly-β-hydroxybutyrate. This is not an unusual feature of *Azospirillum* but had also been described for *Alcaligenes* (Oeding and Schlegel 1973) and *Azotobacter beijerinckii* (Senior and Dawes 1973). Conditions which stimulate the biosynthesis of this polymer are high NAD(P)H, low coenzyme A and high acetylcoenzyme A cellular concentrations, and these conditions are produced during O_2-limitation of growth in *Azotobacter, Alcaligenes* and likely also in *Azospirillum*. When enough respiratory electron acceptors are available, malate can completely by degraded to CO_2 and H_2O, consequently the amount of poly-β-hydroxybutyrate decreases in the cells.

In soils, the possession of a full nitrogen oxide reductase system may be a useful property helping *Azospirillum* to survive under oxygen-limiting conditions, e.g. in waterlogged environments. Generally, denitrification has, however, been considered to be an undesirable feature of nitrogen fixing bacteria like *Rhizobium* (Zablotowicz and Focht 1979; Daniel et al. 1980) and this is probably also the case with *Azospirillum*. When the levels of nitrate are high and those of O_2 are low in soils, denitrification by these bacteria may be a waste of nitrogen lost for plant growth. Most *Azospirillum* strains isolated so far have the capability to perform denitrification, but strains were also isolated which do not dissimilate nitrite and do not produce gas from nitrate (Magalhães et al. 1978a; Tarrand et al. 1978). The latter ones are the strains of the choice, when artificial plant-*Azospirillum* associations are to be constructed.

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